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THE "SINGLE CELL 'COMET' ASSAY" AS A BIOLOGICAL DOSIMETER.

by

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ABSTRACT

The conventional single cell gel electrophoresis, or "comet" assay, uses fluorescence to visualize the distribution of DNA within a "comet" and this, in combination with the comet's tail length, provides a quantitative expression (the tail moment) of the sustained damage. The magnitude of the tail moment is related to the degree of DNA unwinding occurring under alkaline conditions and this in turn depends on the number of DNA strand breaks induced by the radiation exposure. DNA strand breaks represent primary radiation-induced damage and their translation into an overt biological effect (for example, death or mutation) depends largely on the magnitude of the DNA damage and the DNA repair capacity of the cell. In the present study, a variation of the "comet" assay is described which uses a silver stain to permanently visualize "comets" and the apoptotic fraction (rather than tail moment) as a biological endpoint for radiation damage. Apoptosis has several theoretical advantages over DNA strand breaks as the basis of a biological dosimeter for radiation. First, apoptosis is more likely to be detected after an exposure since it can require up to several days to be expressed whereas DNA strand breaks are usually repaired within minutes of being formed. Second, apoptosis can be related to the subsequent development of cancer, in contrast, it is difficult to relate DNA strand breaks to the subsequent development of any overt biological effect. Using manual scoring, dose-dependent changes in apoptotic fraction can be readily detected after *in vitro* exposure to low doses (< 2 Gy) of ionizing radiation. Under the conditions of the silver staining protocol, apoptotic and non-apoptotic cells can be readily distinguished on the basis of their unique DNA distribution patterns or "signatures". These distinctive "signatures" or morphologies can be induced differentially by specific conditions of exposure to toxic chemicals or physical agents such as heat or radiation. The "comet" assay can be applied to any tissue or organ that can be reduced to a suspension of cells. The silver comet assay produces stable images with high signal-to-noise ratios and a unique set of "signatures" which together provide a basis for the development of a fully automated detection system based on inexpensive brightfield illumination. Such a system has the capacity to examine large numbers of cells, an attribute that might be useful in the development of a biological dosimeter.

A. INTRODUCTION

The general objectives of AECB project 7.223.1 were to determine if the apoptotic fraction (AF), as defined by silver stained "comet" assay, could be used (1) routinely as a biomonitor of human exposure to ionizing radiation, (2) experimentally for risk analysis, dosimetry and bioeffects studies in humans or surrogates, such as rodents, and (3) to unravel the mechanisms of radiation action using cells in culture. Specific project milestones, or tasks, were established as follows:

(1) To define dose-response curves for low-LET radiation using human cells (Task 4.1).
(2) To define exposure conditions for alpha particles and neutrons (Task 4.2).
(3) To develop methods for rapid enumeration of exposed cells (Task 4.3).
(4) To develop methods to isolate cells from solid tissues for the comet assay (Task 4.4).
(5) To define dose-response curves for high-LET radiation (Task 4.5).
(6) To evaluate the comet assay as a biological dosimeter using blood as the biological substrate (Task 4.6).

The usefulness of the silver-stained "comet" assay will be evaluated against the dicentric chromosome assay, a biological dosimeter that is widely used for ionizing radiation.

The single cell gel (SCG) or "comet" assay is a sensitive technique for the direct visualization of DNA damage in individual cells. In the conventional protocol, a small number of cells are sandwiched between thin layers of agarose, lysed at either alkaline or neutral pH, electrophoresed, stained with a fluorometric dye and then visualized under a fluorescence microscope. During electrophoresis, loops of DNA migrate away from the nuclear residual to produce a shape with the visual characteristics of a comet. The intensity of the fluorescent stain that migrates to the comet's tail is related to the amount of DNA damage produced in the cell [1,2]. In the alkaline unwinding variant of the SCG assay, DNA single and double strand breaks and alkali-labile lesions are detected while in the neutral variant only DNA double strand breaks are detected. Under neutral conditions, severely damaged cells (those in apoptosis or necrosis) and less damaged cells present with distinctive shapes or "signatures". The proportion of apoptotic cells appear to be directly related to the radiation dose (up to about 2 Gy) whereas the proportion undergoing necrosis may indicate the harshness by which cells were isolated from an organ or tissue or the presence of sub-optimal incubation conditions. It is the neutral variant of the "comet" assay that will be extensively explored in AECB Project 7.223.1.

The SCG assay, as originally described by Ostling and Johanson and developed by Singh *et al.*, has been proposed as a biological dosimeter for ionizing radiation since dose response curves can be obtained down to fairly low doses [3,4]. To obtain satisfactory results however the cells had to be irradiated at ice bath temperatures and assayed immediately to prevent DNA strand break repair. Also, at low doses, the large data set needed to calculate tail moment for individual doses could be limited by photo bleaching of the fluorochrome making it impractical to fully automate the system or provide a permanent record for independent verification of results.

In the neutral variation of the SCG presented here, cells irradiated *in vitro* (or *in-vivo*) are briefly suspended in liquefied agarose, cast as a thin slab on an agarose-coated polyester membrane (™gelbond), and then held at 50°C for one hour in a neutral lysing (DES) solution containing freshly added proteinase-K. The agarose slab is then briefly electrophoresed, fixed, and then air dried to the ™gelbond. The dried film is exposed to a silver stain (Bio-Rad Laboratories) and the comet-like figures are visualized at low magnification using a conventional light microscope. In contrast to fluorometric detection, the permanent record produced by the silver stain allows data to be acquired from a large number of cells without the problems caused by photo bleaching. The dried film can also be stored indefinitely as a record of the experiment either before or after silver staining. To detect apoptosis, the cells must be incubated at 37°C for at least 4-6 hours following irradiation. Under these conditions, cells undergoing radiation-induced apoptosis, form structures with large fan-like tails and small heads, whereas normal or less critically damaged cells form smaller structures with large heads and short, narrow tails, as shown by Olive *et al.*[5] and in figure 1. Cells undergoing necrosis have relatively large nuclear residuals and long, thin extended tails, suggesting random degradation of DNA. In some cells, apoptosis occurs throughout the cell cycle[6] and approaches a maximum value some 24-36 hours after irradiation [7,8].

Measuring apoptosis has several advantages over measuring DNA damage as a biological dosimeter besides being easier to detect. Apoptosis is more likely to be detected after an exposure to radiation since it can appear several days after the exposure whereas DNA damage occurs immediately after exposure and is quickly repaired. Also, since apoptosis is unrepairable, it is more likely to be related to cancer induction than DNA damage. In fact, apoptosis is thought to be a defence mechanism against cancer since it kills cells that are damaged and have the potential to transform into cancerous cells[9].

The use of the comet assay as a biological dosimeter rests on two assumptions; (1) that it measures apoptosis, and (2) that the apoptotic fraction is proportional to the exposure metric. To verify that the comet assay actually measures apoptosis, a comparison must be made between data sets generated by the comet assay and a reference or standardized assay for apoptosis. A strong correlation between the two assays would suggest that both were most likely measuring the same endpoint. For this project, the reference assay, based on cellular morphology, was used to assign cells to one of three categories (non-apoptotic, late stage apoptotic or necrotic). Under the brightfield microscope, a cell undergoing apoptosis, proceeds through a series of well defined morphological changes to a late "stage" in the process characterized by the presence of cytoplasmic condensation, nuclear fragmentation, cell shrinkage, membrane-bound blebs and, eventually, to the formation of small dense apoptotic bodies. A non-apoptotic cell has a clearly defined cytoplasm and nucleus, a circular shape and an evenly stained nucleus (except during mitosis). In a necrotic cell, the membrane becomes porous and jagged and the cell swells (Figure 2)[10]. The apoptotic "signature" generated by the comet assay most likely relates to late "stage" apoptosis, when DNA is being enzymatically degraded into discrete sizes. The strongest correlation should therefore be found when the data set from the comet assay is compared with the data set from late "stage" apoptosis, as defined by the reference assay. The reference method,

while characteristically time consuming and highly subjective, has been widely used to validate other assays that purport to measure apoptosis.

B. MATERIALS AND METHODS.

1. Cell Culture Protocols, Isolation of Cells, Chemicals, and Working Solutions.

1.1 TK-6 Cells

TK-6 cells (American Type Culture Collection, Rockville MD) were grown in RPMI-1640 (Gibco-BRL) medium supplemented with 10% fetal bovine serum (FBS)(Immunocorp Corp., Montreal, PQ) and 2 mM L-glutamine (Gibco-BRL). They were subcultured as needed in 100 mm polystyrene culture dishes and incubated at 37°C in humidified air containing 5% CO_2.

1.2 Human Cells

Blood was collected from humans by venipuncture (7 mL) or finger stab (0.1 mL) into sodium heparinized test tubes or pipettes, respectively. When needed, white blood cells (WBC) were isolated from blood by Ficoll-Paque Plus (Pharmacia Biotech, Uppsala, Sweden) centrifugation at 400 x g for 20 minutes, followed by 3 washes in phosphate buffered saline (50mM phosphate at pH 7.4). Both whole blood (WB) and isolated WBC were irradiated *in vitro* at various doses (up to 2 Gy), incubated at 37°C for various times in tissue culture medium (consisting of RPMI-1640 medium supplemented with 20% v/v heat inactivated FBS, Immunocorp, Montreal PQ, 2 mM L-glutamine, Gibco-BRL, 100 IU/mL penicillin and 100 µg/mL streptomycin, Gibco-BRL). Human cheek epithelium was scraped from the inside of the mouth with a wooden applicator stick and the scrapings washed into tissue culture medium (supplemented with 200 IU/mL penicillin and 200 µg/mL streptomycin) and incubated at 37°C [11-13].

1.3 Mouse Cells

A variety of mouse cells were examined as potential substrates for the "comet" assay. Mouse blood was isolated by cardiac puncture and collected into sodium heparinized test tubes or syringes. Bone marrow was flushed from the femur cavity with ice cold tissue culture medium. Spleen and liver were isolated, washed free of blood, and then finely minced with scissors or a scalpel blade. The brain was removed intact and finely minced with scissors and the nuclei were isolated according to the method of Hymer and Kuff [14]. Testes were removed, triturated with 3 strokes of a Dounce style tissue grinder and then suspended in ice cold tissue culture medium.

1.4 Chemicals and Aqueous Solutions

Aqueous solutions were made with 18 MΩ water (Milli-Q plus PF unit, Millipore Corp., Bedford, MA). All glassware and plastic coated magnetic stir bars used in the preparation of the silver stain were pre-washed with 50% nitric acid, followed by multiple sequential rinses in distilled water and 18 MΩ water. A 1% w/v agarose solution (Bio-Rad, high strength #162-0125, Hercules, CA) was prepared in Hanks balanced salt solution (HBSS). The DES lysis buffer (lithium chloride 1 M., urea 1 M., EDTA 10 mM., Tris base 50 mM., SDS 2% w/v - all from Fisher Scientific, Fairlawn, NJ) was prepared at pH 7.3, filtered (0.2 µm filter, Nalge Company, Rochester, NY) and then stored at ambient temperature until needed. Immediately before use, proteinase-K was added to the lysis buffer to a final concentration of 60 µg/mL. A proteinase-K stock solution (Boehringer-Mannheim Corp., Indianapolis, IN) was prepared at 20 mg/mL in TE buffer (Promega Corp. Madison, WI), filtered (0.2 µm pore size) and stored in 1 mL aliquots at -30°C. The electrophoresis was run in a TAE buffer (Tris-Acetate 0.04 M., EDTA 0.01 M., glacial acetic acid 0.02 M., Boehringer Mannheim). Trichloroacetic acid (Fisher Scientific, Fair Lawn, NJ) was prepared as a 20 % w/v solution and filtered (0.2 µm pore size, Nalgene). ™Silver stain plus kit (Bio-Rad, Hercules, CA) was used, with modified protocol, as a permanent stain for the "comets". Methanol and glacial acetic acid were from Fisher Scientific. Giemsa stain (EM Diagnostic Systems, NJ) was prepared as a 5% v/v solution in balanced salt solution buffered at pH 6.9. Tritiated water (5 Ci/mL) and the ^{131}I (40 Ci/mL) was obtained from Amersham Canada (Oakville, ON) and ^{241}Am was supplied by Analytics Inc., Atlanta GA.

2. Radiation Exposures

2.1 External Irradiation

Immediately before irradiations, TK-6 cells were collected by centrifugation (400 x g for 5 min.) and resuspended in fresh, ice cold tissue culture medium at no less than 2.5×10^5 /mL. Whole blood or isolated white blood cell suspensions were diluted with tissue culture medium to give at least 5×10^5 white blood cells/mL. Cells were exposed at 0°C to $^{60}Cobalt$ gamma rays (1.07Gy/min), to 190 kVp (0.17 Gy/min, HVL=8.8 mm Al) or to 250 kVp X-rays (0.53 Gy/min, HVL=1.75 Cu). A Phillips Industrial unit operating at 190 kVp (effective energy 57.5 keV) and 5 mA was used at the Radiation Protection Bureau. Source to chamber-centre distance was 35.0 cm and the field size was 12.5 cm x 12.5 cm. A Radcal pencil chamber (Model 2025-10.3 CT) with calibration traceable to the national standards laboratory was employed as the dose meter. Dose rate was predetermined at the central axis prior to each irradiation. The Radcal monitor was placed 4.0 cm on the left of the central axis to record cumulative exposures from which the actual dose was determined. Samples were positioned on the central axis on the table top. Overall uncertainty in the actual dose delivered was estimated at 5.5% (beam flatness (± 1.5%); off axis ratio (± 1.0%); detector calibration and readout (± 5.0%). The exposure rate (ER) in R/min was calculated as follows:

$$ER = \frac{R \times C \times T}{P} \quad (1)$$

where: R = radcal reading
C = calibration factor = 1.02
T = temperature in °C
P = pressure is in kPa

The dose rate (DR) in Gy/min at the central axis was calculated as:

$$DR = ER \times f_{med} \quad (2)$$

where: ER = exposure rate at the central axis
F_{med} = conversion factor for water = 0.00908 Gy/R

The other X-ray units were similarly calibrated. The ^{60}Cobalt source was an Eldorado-6 unit (Atomic Energy of Canada Ltd.) located at the National Research Council (Building M-35, Montreal Rd. Campus).

After irradiation, the TK-6 cells were incubated at 37°C (5% CO_2) for 24 and 48 hours before being embedded in agarose and processed through the comet assay while the blood cells were incubated for 6, 24, 48 and 72 hours.

2.1.1 External Irradiation of Mice by 120 kVp X-rays

C57BL/6 male mice (15-24 g), acquired at 5 weeks of age from Charles River, Canada Ltd. (LaSalle, PQ) were allowed to acclimatize for one week under housing and feeding protocols approved by the Animal Care Committee of Health Canada. Each mouse was put into a 50 mL centrifuge tube (3 mm holes were drilled in each tube for air circulation). The mouse holder was placed at the centre of the top shelf of the X-ray unit, aligned with the X-ray beam and exposed for an appropriate time. The X-ray generator was a Dinex model 150R, serial no. 1071 (Torr X-ray Corp, van Nuyes CA) which gave 120 kVp X-rays at 5 mA at a calculated dose rate of 0.647 Gy/min. The mice were sacrificed 3 h to 2 weeks post irradiation and the required tissue was collected.

2.2 Radionuclides and Exposure Protocols

Cells at $2x10^5$/mL were exposed for 24 h at 37°C to ^3H_2O by adding different volumes of a 0.1 mCi/mL ^3H_2O stock solution to 5 mL of tissue culture medium, supplemented with 20 mM HEPES at pH 7.3. After 24 h cells were collected by centrifugation (400 x g for 5 min.) washed in fresh medium, transferred to 100 mm culture dishes, incubated at 37°C for up to 24 or 48 h and

then harvested for the "comet" assay. Cells were exposed to ^{131}I for 24 h on ice and then incubated for 24 h at 37°C. Cells were also exposed to ^{241}Am by adding varying amounts of a 10 μCi/mL ^{241}Am solution to 1 mL of RPMI-1640 and incubated for 24h at 37°C before being harvested for the comet assay. The dose to cells was estimated to be 3.2 Gy/h per mL of $^{3}H_2O$ added and 1.2 Gy/h per mL of ^{241}Am added. Due to technical difficulties, the cells were not exposed to ^{32}P as originally planned.

A ^{222}Rn exposure system was developed in which single cell suspensions of TK-6 cells were to be irradiated by ^{222}Rn and its progeny. The system used a $RaCl_2$ source which produces radon gas. Air was pumped through the source and into a tissue culture vessel containing a suspension of cells. The ^{222}Rn concentration in the air was continuously monitored by a 160 mL scintillation cell along with a photomultiplier tube which was attached to a computer for data acquisition. Grab samples were also taken in 160 mL scintillation cells and measured with a calibrated photomultiplier tube to assess the exact radon concentration in the air. The amount of ^{222}Rn absorbed in the tissue culture medium was measured using a liquid scintillation counter. A diagram of the exposure system is shown in **Appendix I**. The system was tested and found to be operational but unfortunately, before it could be applied to cells in suspension, the room had to be vacated and the system dismantled because of management's need for space.

2.3 Neutron Exposure

Neutron exposures were carried out at the Royal Military College in Kingston ON with the assistance of the Department of National Defence staff at Shirley's Bay (Tom Cousins). Dosimetry was predetermined by the use of rhodium foils and also monitored during actual exposure using calibrated rhodium foils.

TK-6 cells and human blood was transported to Kingston on ice, irradiated, and transported on ice back to the Radiation Protection Bureau for analysis.

3. Cell Viability

Cell suspensions were assessed for number and viability using a modification of the technique described by Hartmann *et al.*[15]. Viabilities were done every time a sample of cells was cast in agarose for the "comet" assay. Briefly, 2 stock solutions were prepared; fluorescein diacetate at 5 mg/mL in acetone and ethidium bromide at 200 μg/mL in Hank's Balance Salt solution (HBSS). A working solution of the dye was prepared in a small glass test tube by mixing 30 μL and 200 μL of the fluorescein diacetate and ethidium bromide stock solutions respectively, with 4.8 mL of HBSS. Equal volumes (usually 30 μL) of working dye solution and cell suspension were pre-mixed in a small glass test tube and then 10 μL was placed on the chamber of a hemocytometer and examined with a fluorescence microscope (x10 or x20 objective) at an excitation of 545 nm and an emission of 610 nm. For viability, dead cells appeared red and live ones green. Cell number was assessed on the same grid under tungsten illumination and phase contrast optics.

4. Single Cell "Comet" Assay

™Gelbond (FMC Bioproducts, Rockland, ME) was cut into 6.4 x 9.9 cm strips to fit an agarose C gel mould (part no. HE-4710, Hoefer Scientific Instruments, San Francisco, CA) and then the ™gelbond-containing mould was placed into a casting tray (part no. HE33-B, Hoefer Scientific Instruments). To prevent curling of the ™gelbond rectangle, the long edges were secured in the mould by 2 thin teflon strips (15 x 65 x 1 mm, Bio-Rad, Hercules,CA) so that the 1 mm edge forced the ™gelbond sheet to lie flat against the bottom of the mould. 1.0-1.5 x 10^5 cells suspended in 0.2 mL of HBSS was added to 2.8 mL of prewarmed agarose (50° C) in (2.5x15 cm) glass tubes and the cell-agarose suspension was briefly mixed with a 5 mL pipette and spread onto the ™gelbond. At these dimensions, 3.0 mL of agarose-cell suspension produced a layer 0.7-0.8 mm thick. The length of time the cells remained at 50°C was less than 10 seconds. When the agarose was fully solidified (~5 min), the gel-™gelbond slab was carefully transferred from the casting tray to a tissue culture dish (150mm) and incubated at 50°C for one hour in 50 mL of DES lysing solution containing 60 µg/mL of proteinase-K. After lysis, the gels were washed twice for 20 minutes each in TAE running buffer and transferred to submarine agarose gel units (model HE-33, Hoefer) for electrophoresis at 22 V for 5 minutes (Gibco-BRL Power Pack model 4001, NY). Gels were transferred back to their 150 mm culture dish, washed with three brief changes of 18 MΩ water, fixed in absolute ethanol for 1-2 hours and dried to a thin film overnight at ambient temperature or on a slide warmer set at 39°C. The film was divided in two parts, one for archiving and the other for silver staining. The thin dried agarose sheet was stained according to the suggestions of the kit manufacturer (™Silver Stain Plus, Bio-Rad Laboratories Ltd., Hercules CA), but with the following modifications. After drying, the film was washed for 1 hour in a solution consisting of 9 parts TCA (20% v/v) to 1 part of ™Fixative Enhancer (Bio-Rad), a component of the kit, followed by a 20 minute wash in water. The silver stain solution was prepared in subdued light from the kit components according to the manufacturer. Briefly, 25 mL of staining solution was added to the dried gel film in a 100 mm culture dish, and the dish agitated for 9 to 14 minutes in a gyratory bath set at 60 cycles/min until adequate staining of the cells was observed under light microscopy. To stop the staining process, the gel was removed from the stain and immersed for 20 minutes in an stopping solution of methanol: glacial acetic acid: water (50:16:34) and finally rinsed in water.

Overstained comets could be readily destained by following the procedure of Merril et al.[16]. The films were briefly air dried and mounted with scotch tape on 4 x 5 inch glass slides (Kodak Ltd.) for analysis under the light microscope.

5. Detection of Apoptosis Using a Reference Assay Based on Cell Morphology

For morphological assessments, TK-6 cells were treated with 0-10 µM hydrogen peroxide (H_2O_2) for 8-18 h to induce apoptosis. 150 µL of cells at a concentration of 2.5-8 x10^5 cells/mL were placed on top of 125 µL of RPMI containing 30% FCS in a cytocentrifuge cell holder and centrifuged at 1000 rpm for 6 minutes. The slides were immediately fixed in a 3:1 solution of ethanol and glacial acetic acid for 1 min, placed in 50% ethanol for one minute, allowed to air dry and stained in 5% Giemsa stain for 5-10 minutes.

6. Data Collection and Statistical Analysis

6.1 Comet Assay Data Collection

6.1.1 Manual Data Collection

Data for estimating the apoptotic fraction (AF) produced by a particular dose was acquired through a brightfield microscope (Olympus BH-2) with a x10 objective lens (Olympus apochromat, NA=0.4). An observer can visually detect comets with 3 distinct morphologies; normal (elliptical shape, short tail), apoptotic (large pear shaped) and necrotic (large head and long thin tail) (figure 1). Necrotic cells were rarely observed after low doses of radiation, but could be readily produced by prolonged exposure to 50°C, fixatives such as ethanol:glacial acetic acid (3:1) or high concentrations of hydrogen peroxide (H_2O_2). The AF was expressed as the ratio of the number of apoptotic figures (A) to the total number of figures observed n+A (n is the number of cells with "normal" morphology). On average, 1000 cells were counted on each slide

6.1.2 Automated Data Collection

With the comet assay, the lengths and widths of a limited number of representative apoptotic and non-apoptotic cells were measured with an ocular micrometer calibrated in μm against a stage micrometer (Olympus, OBM1/100). Areas and perimeters were estimated using the Matrox Imaging Library software, in combination with a Matrox Comet Video card (Matrox Electronic Systems, Dorval, Quebec, Canada). For imaging, the BH-2 was fitted with a 3.3 x photoeye piece, an 8 bit CCD monochrome camera (Cohu 4810 series with 640 x 480 pixel resolution) and a x 0.55 zoom lens. Final image resolution was about 5.76 μm^2 per pixel. Comet recognition software was developed to differentiate between normal and apoptotic cells, however some limitations still exist. The system automatically steps through the microscope slide and refocuses on every fifth field of view. By measuring the perimeter of each cell, the program discriminates between normal and apoptotic cells according to a predetermined threshold for each cell type. A problem occurs, however, when a cell which is clearly apoptotic to the observer (by comet morphology), is smaller than the threshold and is counted as normal. This program was found to have a high percentage of false positives and false negatives and the apoptotic fraction, as measured by the computer, rarely matched that of the manual observer. The software, however, was modified to measure the distribution of DNA in the head and tail of the comet and the tail length which allows the observer to calculate the tail moment. This measure of DNA damage under alkaline (rather than neutral conditions) may correlate better with dose, under limited circumstances, than does the apoptotic fraction. The alkaline comet assay is currently being tested on the brains of mice exposed to x-rays, and will be attached as an addendum to this report when completed.

6.2 Giemsa Stain Data Collection

After Giemsa staining, the morphology of the cells were examined through a brightfield

microscope with a 100x oil objective lens. The 3 morphologies could be visually distinguished as can be seen in figure 2. Cells were counted as either normal or late stage apoptotic. Again, necrotic cells were rarely observed at the low concentration of H_2O_2 (used as a radiation surrogate) or after low doses of radiation but became apparent when cells were exposed to high concentration of H_2O_2 or high doses of radiation.

6.3 Statistical Analysis

Statistical analysis was done according to methods published in Ipsen and Feigl,[17]. Briefly, the true rate of apoptosis in TK-6 cells after exposure to ionizing radiation was estimated by p = A/N, where A is the number of apoptotic cells and N is the total number of cells examined (n+A) at time t. For N observations, the variance of rate p is defined as $\sigma^2 = pq/N$, where q = (1- p). The error bars on the graphs for one representative experiment show the standard deviation of each point calculated from the variance. When several experiments were averaged, the error bars represent the standard error of the mean (SEM) between the experiments.

C. RESULTS

1. Assay for Cell Morphology

The validity of the comet assay as a detector of apoptosis was tested against the results from an an accepted standard which uses Giemsa stain to detect apoptosis on the basis of morphological criteria. To compare the two assays, TK-6 cells were treated with 0-10 μM of H_2O_2 (a radiomimetic chemical) in order to induce apoptosis. The cells were analysed at different time points (8-18 h) using both assays. In figure 3, representative data averaged from two experiments show a correlation between apoptosis measured by the comet assay and from cell morphology after 8 hours of exposure to H_2O_2. It can be seen that there is a strong correlation between the two methods for detecting apoptosis (R^2=.998). The slope of 0.881 indicates that the comet assay detected slightly more apoptotic cells than did the reference method. The correlation coefficients between the two methods were similar at all time points (data not shown). Little or no necrosis was detected with either method indicating that the comet assay is not confusing apoptotic cells with necrotic cells.

2. Dose Response of TK-6 Cells

Examples of apoptotic and non-apoptotic TK-6 cells are shown in Figure 1. Using ™Matrox image analysis software and brightfield optics (x10 objective lens, Zoom set at x0.55 and a x3.3 photoeye piece), the area and perimeter of representative apoptotic cells were, respectively, 26,000 +/- 6000 μm^2 and 700 +/- 80 μm, while for non-apoptotic cells the dimensions were, respectively, 14,000 +/- 4000 and 490 +/- 60 μm. Necrotic cells had areas approximately equal to apoptotic cells but shape, not area, was considered the important distinguishing parameter.

Human TK-6 cells were used to establish initial dose-response relationships and to define the change in AF with dose after 24 hours of *in vitro* incubation, as shown in Figure 4 for 190 and 250 kVp X-rays and ^{60}Co gamma rays, in Figure 5 for $^{3}H_2O$, ^{131}I and ^{241}Am and in Figure 6 for neutrons after 48 hours of incubation. A dose-dependent increase in AF could be readily detected for all types of radiation in the range of 0 to 2.0 Gy. After 250 kVp X-irradiation, a greater response was attained after 48 hours as compared to 24 hours of incubation at 37°C (Figure 7). The rate of change in the apoptotic fraction Gy^{-1} (Δ AFGy $^{-1}$) for each type of radiation, except ^{131}I, was estimated for TK-6 cells using a linear fit to the appropriate dose-response curve. The ^{131}I data clearly was not linear and therefore was not included in the comparison of different types of radiation. For acute exposures, Δ AFGy $^{-1}$ was greatest for the 190 kVp X-rays and decreased in the order of 250 kVp X-rays and ^{60}Co.This order is in agreement with the order of LET of the irradiations with the lower energy X-rays having the highest LET and the ^{60}Co having the lowest LET. The Δ AFGy $^{-1}$ for ^{241}Am was 0.181, and 0.012 for $^{3}H_2O$. This data fits in with the photon data with respect to LET and $\Delta AFGy^{-1}$. From this data, an estimate was made of the number of cells that would need to be examined in order to measure a significant difference between the treated and untreated cells. The sample size needed to detect a statistically significant increase of 0.025 in AF over an unexposed control is shown in Table 1 for α and β errors of 0.05. Typically, the background AF for unexposed TK-6 cells was 0.014 +/- 0.002 in 8 experiments. From this Table it can be seen that for lower LET radiation, very large numbers of cells need to be counted in order to detect small differences in dose received.

3. Dose Response of Human Blood Samples

The results from *in vitro* irradiation with 190 kVp X-rays of whole blood cultures from a "responding" donor is shown in Figure 8. For unexposed control blood, the AF increased steadily from 0.018 at the start of the experiment to about 0.210 at 12 hours, reaching a plateau at about 0.24+/- 0.03 after 12 hours of *in vitro* incubation. For irradiated blood, the increase in AF was non-linear over time, reaching a plateau by 30 hours. As shown in the insert in Figure 8, after a 30 hour incubation, the AF increased linearly with dose. In another experiment using 250 kVp X-rays, the time course of apoptosis in WBC was compared under two different *in vitro* conditions of irradiation and incubation. In the first, whole blood was exposed to 2 Gy and incubated for various times before assessing the AF of WBC *in-situ*. For these WBC, the AF reached a maximum of 0.34 at 72 hours (Figure 9). In the second, WBC were first isolated from whole blood, exposed to 2 Gy and incubated in tissue culture medium before being assessed for apoptosis. For isolated WBC, the AF reached a maximum of 0.45 at 48 hours (Figure 9). In some *in vitro* experiments, cells maintained at < 200,000/mL failed to respond to radiation (data not shown), consequently, all experiments involving isolated WBC were run at cell concentrations of > 250,000 /mL. Once it was established that apoptosis could be measured in whole blood obtained from venipuncture, the next goal was to determine if this method could be used on whole blood obtained from a finger stab. Figure 10 shows the apoptosis as a function of time of blood irradiated *in vitro* from the same donor. The blood was obtained by either finger stab, venipuncture or WBC separated from the venipuncture blood. All three preparations of blood responded similarly over time, however, the WB taken directly from the finger stab had the

highest fraction of apoptosis after 48 hours. This indicates that WB taken directly from a finger stab, without any processing, could be useful for measuring apoptosis.

Despite adequate cell concentrations and controlled irradiation and incubation conditions, the WBC of some blood donors responded poorly, or not at all, to radiation (Figure 11). Even the same donor did not always respond to the same extent as can be seen with WBC from donor 1 which had a high response after 2 Gy but a much lower response after 5 Gy. The time of incubation to attain maximum apoptosis in isolated WBC was fairly predictable, being 48 hours for most donors, as can be seen in Figures 7 and 8, however, the WBC from one donor (donor 2) reached maximum apoptosis after 24 hours (Figure 11). The AF in the WBC of non-responders remained uniformly low over the entire 72 hour incubation period without a discernible dose-response relationship for the irradiated sample (Figure 11, donor 3).

4. Dose Response After *In Vivo* Exposures of Mice to X-rays

Mice were irradiated with X-rays at doses of 0.5, 1.0 or 3.0 Gy and then held for 6 to 336 hours before cells were harvested for analysis from blood, bone marrow, testes and brain. The brain was reduced to a suspension, nuclei were isolated from this suspension into tissue culture medium and then processed immediately through the "comet" assay without a period of *in vitro* incubation. Blood and bone marrow suspensions were assessed immediately for apoptosis without a period of *in vitro* incubation. For blood, two mice were sacrificed for each dose and time point. There was no induction of apoptosis after any dose at any time point except for one mouse which had an AF of 0.206 24 hours after a 3 Gy exposure (Figure 12). The same result was observed on the mouse testes, with the same mouse being the only responder in the treated group (Figure 13). Neither the bone marrow nor the brain cells responded to the irradiation (data not shown). In another experiment that did not involve X-irradiation, the apoptotic fraction in spleen and skin were both at a low level suggesting that these organs could be potential substrates for future radiation studies.

D. DISCUSSION.

The objective of this project was to determine if apoptosis, as measured by the silver-stained "comet" assay, could be used as a biological dosimeter at low doses (< 1 - 2 Gy) of ionizing radiation. A biological dosimeter can be used to (1) monitor individuals for occupational or accidental exposures, (2) in risk analysis to study the effects of radiation on human surrogates (rodents, monkeys) or (3) experimentally with cells in culture to study mechanisms of action. The "comet" assay was evaluated to determine its potential to meet all or some of these objectives.

Using TK-6 cells, the AF was found, in agreement with others [18,18-21], to increase in a linear

dose-dependent manner up to about 2 Gy. Therefore, the comet assay has some potential as a biological dosimeter for low doses of ionizing radiation. To be considered useful in this regard, the "comet" assay must have attributes that equal or exceed those of an accepted biological dosimeter, such as the dicentric chromosome assay (DCA). The dicentric chromosome is (almost) an exclusive marker for radiation exposure with a background level in the general population of about 0.001 dicentrics/cell. White blood cells respond the same to radiation regardless of whether they are exposed *in vitro* or *in vivo*. In addition, there is a relatively low order of variability in the dicentric yield for a specific type of radiation within and between donors and the yield remains unchanged in the peripheral blood for up to a year[19]. Despite these positive attributes, the DCA assay has a subjective component with observer error and bias adding uncertainty to dose estimates. An additional downside is the lack of a fully automated analytical system, although metaphase chromosome finders do exist (see "Improved Cytometry Method, AECB project 7.164.1).

The ideal biological dosimeter should be based on techniques that are simple to perform, rapidly deployable in the case of accident or emergency, sensitive, dose-dependent and reproducible. The "comet" assay is simple in that it can be run directly on blood obtained by finger stabs or in a solid tissue that has been reduced to a suspension of cells. It is also relatively easy to process many samples simultaneously.

Before determining whether the "comet" assay could be used as a biological dosimeter, it was important to confirm that the cells identified as apoptotic by the "comet" assay were actually apoptotic cells. Giemsa stain, which allows the morphology of the cells to be examined under light microscopy, was used as a reference method to validate the "comet" assay. When all cells were undamaged, both methods identified the cells as normal or non-apoptotic. When H_2O_2 a radiomimetic chemical, was used to treat the cells, both methods identified the damaged cells as apoptotic and not necrotic. The "comet" assay, however, measured a slightly higher percentage of apoptotic cells than was found by the reference method. The strongest correlation occurred when the late stage of apoptosis, as defined by morphological assessment, was compared with the apoptotic fraction as defined by the "comet" assay. The correlation between apoptosis measured by the two methods was, however, extremely good. From these results, it can be concluded that the "comet"assay is able to distinguish between apoptotic, necrotic and undamaged cells.

The results shown here demonstrate the sensitivity of the assay at low doses (<2 Gy). When 1000 cells are counted manually, the assay can theoretically measure to an accuracy of ±0.1% but taking into account operator error in counting, a more realistic estimate of the accuracy would be ±0.5%. This translates into being able to detect damage from doses as small as 0.2Gy. A dose response can be seen for all types of radiation and each type produced a different response. These responses are summarized in Table 1 in order of decreasing LET where the alpha emitter (^{241}Am) produced the most apoptosis and this decreased down to the beta emitter ($^{3}H_20$) which produced the least.

Preliminary results with the TK-6 cells suggest that the rate of apoptosis at a specified time after

exposure, as measured by the "comet" assay, may have some potential as a biological dosimeter for ionizing radiation. These dose response curves are reproducible as can be seen by the error bars which represent the SEM of at least 3 experiments. Table 1 also shows the number of cells that need to be examined and classified to detect a difference of 0.30 Gy between the exposed and unexposed cells. For the lower LET irradiation, these numbers are large enough to make manual enumeration impractical. In contrast, automation would allow small differences to be detected at the required level of statistical significance. The silver stain facilitates the automation of cell evaluation by providing a permanent image of the cells. Dried stained or unstained gels can be stored for 3 or more years without apparent deterioration. Subsequent software development will concentrate on distinguishing apoptotic and non-apoptotic on the basis of morphological parameters and not predominantly on the basis of size.

In contrast to human TK-6 cells, freshly isolated human white blood cells (WBC) and whole blood (WB) irradiated *in vitro* gave more variable and unpredictable results. A blood donor could be classified either as a "responder" or a "non-responder" to radiation. For a "responder", the AF in a sample of unexposed WBC increased steadily over a 24 hour post-irradiation incubation period and reached maximum AF which remained steady until 48 h. For irradiated cells, the AF increased faster than in unexposed cells and reached a higher plateau. The maximum AF (measured at 30 h) increased with dose. Another pattern was observed when the AF in irradiated WBC and WB samples were compared over 4 days of incubation at 37°C. The AF for irradiated isolated WBC reached a maximum at 48 hours whereas the AF in whole blood peaked at 72 hours; the AF of the unirradiated samples remained low, never exceeding 0.20. The AF of a responder using WB from venipuncture and finger stab and isolated WBC in tissue culture medium was also compared after 2 Gy over 96 h. Similar trends were seen with each blood cell isolation technique. The finger stab gave the highest response, which might be the result of blood handling. The finger stab method of blood collection was the harshest and may have contributed to the observed higher rate of radiation-induced apoptosis or to a cell's susceptibility to apoptose after irradiation. In any case, blood collected by finger stab gave a good response to radiation and may have some potential as a sampling source for biological dosimetry. The variation in the AF for the blood of responders can be seen in figure 11. The AF for an apparent non-responder after 5 Gy of radiation remained uniformly low over the entire 72 hour incubation period without showing any discernible radiation dose-response relationship. Under the same conditions, the AF of a responder reached 0.40 after just 2 Gy. The AF in the blood of an intermediate responder reached 0.17, but at an earlier time point. These data suggest that the response of the WB to radiation varies from person to person in both maximum AF and the time at which this maximum is reached. These results indicate that the use of the "comet" assay to monitor individuals for occupational or accidental exposures might only be feasible if a pre-exposure WB sample was available Since this is not likely to be the case, the use of the "comet" assay as a radiation dosimeter may not be feasible because of the inter-donor variation in response to ionizing radiation.

Limited experiments with skin and cheek epithelium suggests that most of the cells were apoptotic upon isolation and were not be suitable for biological dosimetry. This is understandable

since the outer layers of the skin normally undergo apoptosis prior to sloughing off as dead skin or squames.

Our results, however, suggest the "comet" assay can potentially meet the second and third study objectives. The results from the mouse experiments show little apoptosis in the blood and testes for all dose and time points except for the one mouse which responded after 24 h and 3 Gy. It is believed that the lack of apoptosis is due to the apoptotic cells being quickly phagocytosed by the macrophages *in vivo*. This would mean that the apoptosis would be difficult to measure *in vivo* since the apoptoic cells would not accumulate as they do in the *in vitro* situation. The one mouse that did have a high AF was either very sensitive to radiation or the time of sampling coincided with the time of apoptosis. No apoptosis was seen in the mouse brain cells at any time.

The experiments on TK-6 cells gave reproducible dose response curves, indicating the use of the "comet" assay in these contexts could be used in investigating mechanisms of interaction between ionizing radiation and chemical radioprotectors or radiosensitizers, or to investigate the effect of secondary risk factors in radiation protection.

This assay also has the possibility of being able to meet another objective of radioprotection; that of measuring an individuals radiosensitivity. The assay appears to distinguish between individuals with varying degrees of response to an irradiation and those that are "non-responders". Such individual variations may correlate with an individual's radiosensitivity. Further studies will be conducted in the future to examine this correlation in greater detail.

By using the apoptotic fraction as a measure of radiation response, rather than DNA strand breaks or chromosome aberrations, the analysis becomes quick and straightforward, simply requiring the observer to distinguish between the apoptotic and non-apoptotic figures as opposed to calculating the tail moment for each cell as in the traditional "comet" assay, or making detailed chromosome counts for each cell as required by the DCA. Preliminary development of automation software suggests the possibility that large amounts of dosimetry data can be acquired quickly and be used to discriminate between low doses.

The "comet" assay can detect apoptosis with a high degree of sensitivity, but the apparent variability in the apoptotic response of each individual to ionizing radiation precludes its use as a practical biodosimeter. The variability in response to radiation however could, in part, reflect the influence of the biochemical rhythms that underlie all living creatures or certain unidentified lifestyle factors, or, more probable, the individual's genetic makeup. Therefore, it may be possible for the "comet" assay to measure, or predict, an individual's radiosensitivity. This possibility will be explored in a set of future experiments, the results of which will be made available to the Board. The "comet" assay appears to have some immediate use as a research tool to investigate mechanisms of action and interaction of ionizing radiation with other physical agents (heat, UV light etc.), drugs (radioprotectors and radiosensitizers) and toxic environmental chemicals.

TABLE 1: The change in apoptotic fraction Gy^{-1} (Δ AF Gy^{-1}) in TK-6 cells 24 hours after exposure to low doses of various types of ionising radiation. N is the sample size needed to detect a difference of 0.30 Gy between exposed and unexposed cells (α=0.05 and β=0.05). The AF of unexposed cells was consistently observed to be about 0.015 or less.

Radiation Type	Δ AFGy^{-1}	Sample Size (N)
^{241}Am	0.181	400
190 kVp X-rays	0.0809	1250
250 kVp X-rays	0.0658	1700
$^{60}Cobalt$	0.0333	5300
$^{3}H_2O$	0.0117	35000

a.

b.

c.

Figure 1: Silver stained comets of TK6 cells. Figure 1a shows a non-apoptotic cell, figure1b shows an apoptotic cell and figure1c shows a necrotic cell.

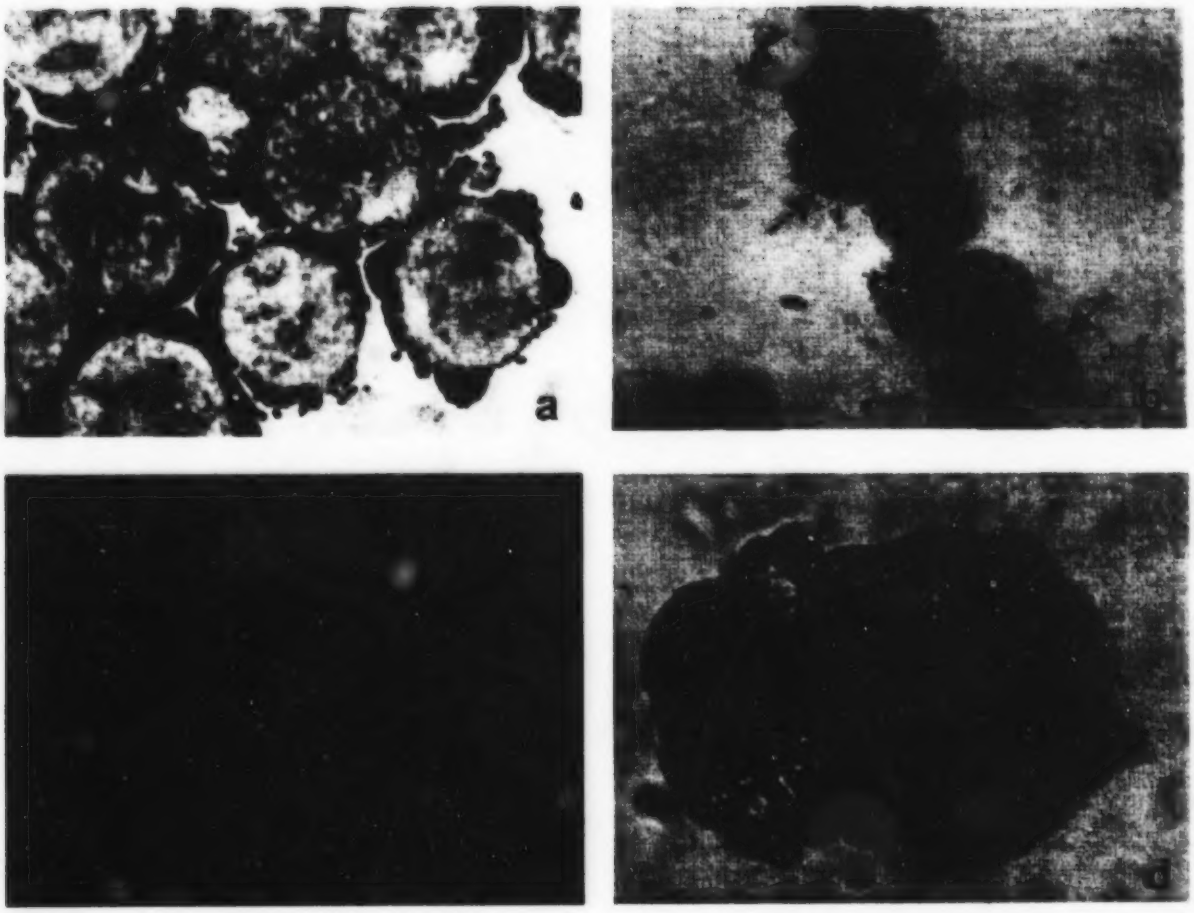

Figure 2: Cell morphology after Giemsa staining. a)apoptotic cell (arrow) surrounded by normal cells. Note the nuclear fragmentation and cell shrinkage. b) apoptotic (arrow), normal and necrotic (N) cells. Note the two cells which have just undergone division. c)Late apoptotic cell. Note the membrane blebbing and breaking into apoptotic bodies. d) Apoptotic cell and necrotic cell.

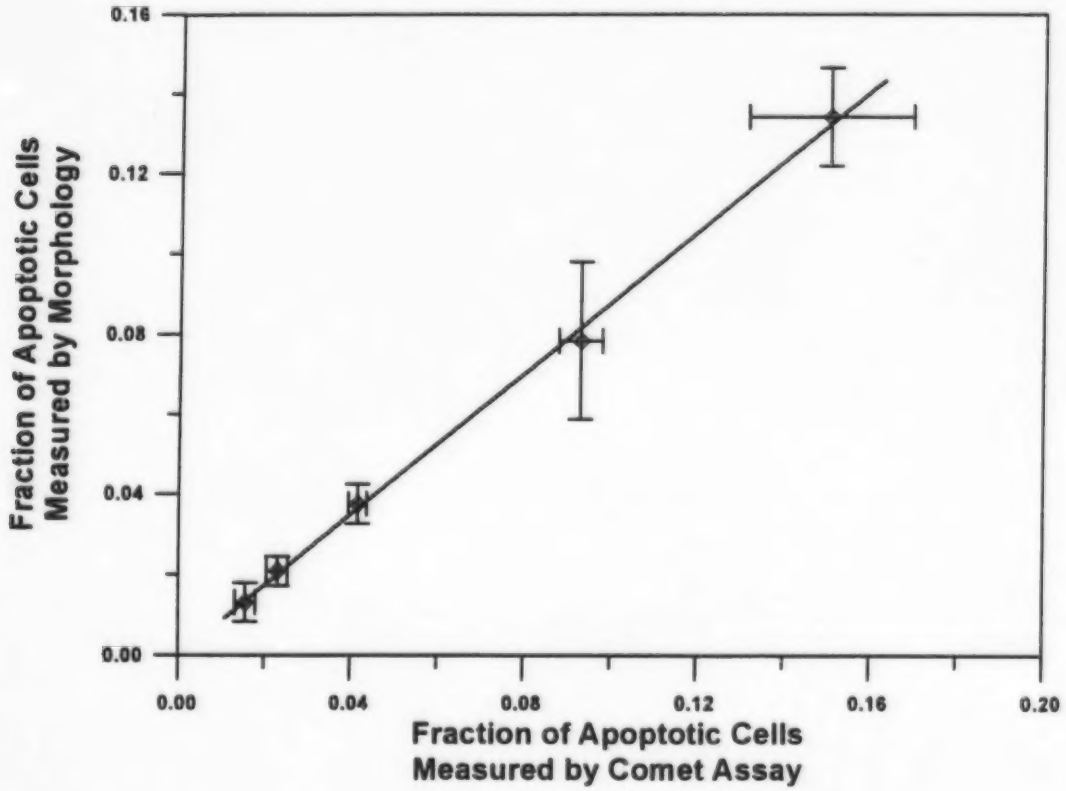

Figure 3: Fraction of apoptotic TK-6 cells as measured by the Geimsa stain vs. the "comet" assay after incubation with increasing concentrations of H_20_2 for 8 h.

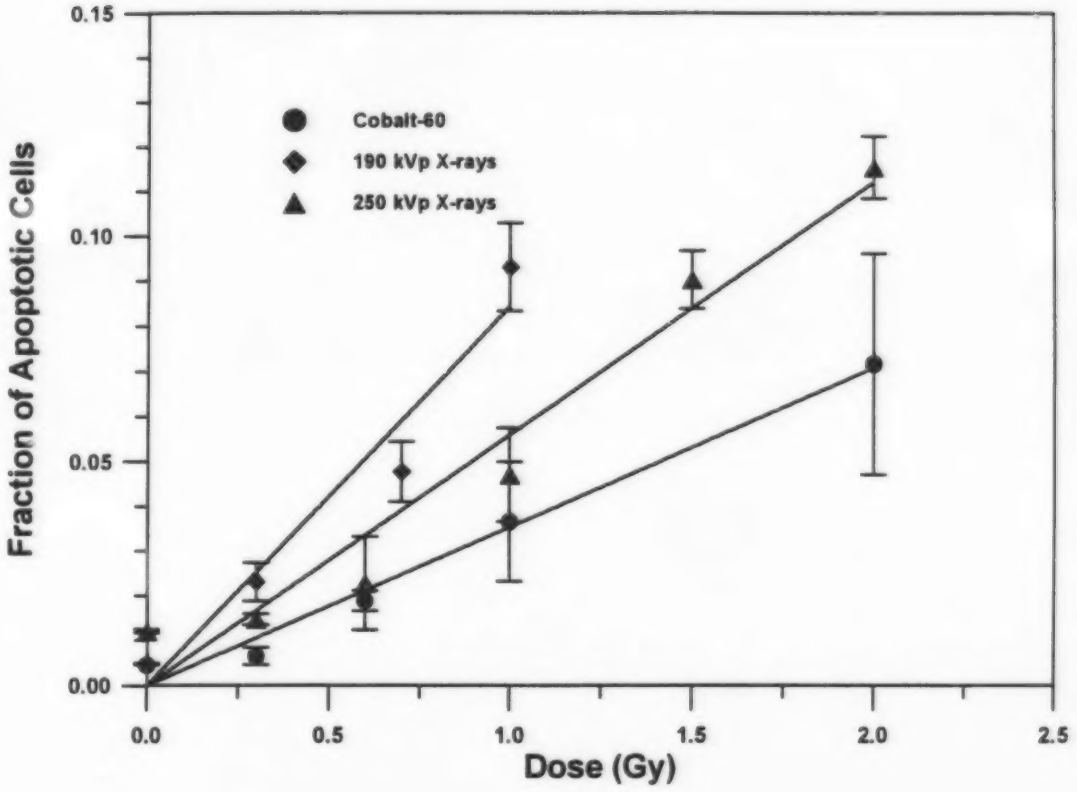

Figure 4: Fraction of apoptotic TK-6 cells 24 hours after ^{60}Co, 190 kVp X-rays and 250 kVp X-rays. Error bars represent the standard error of the mean between experiments.

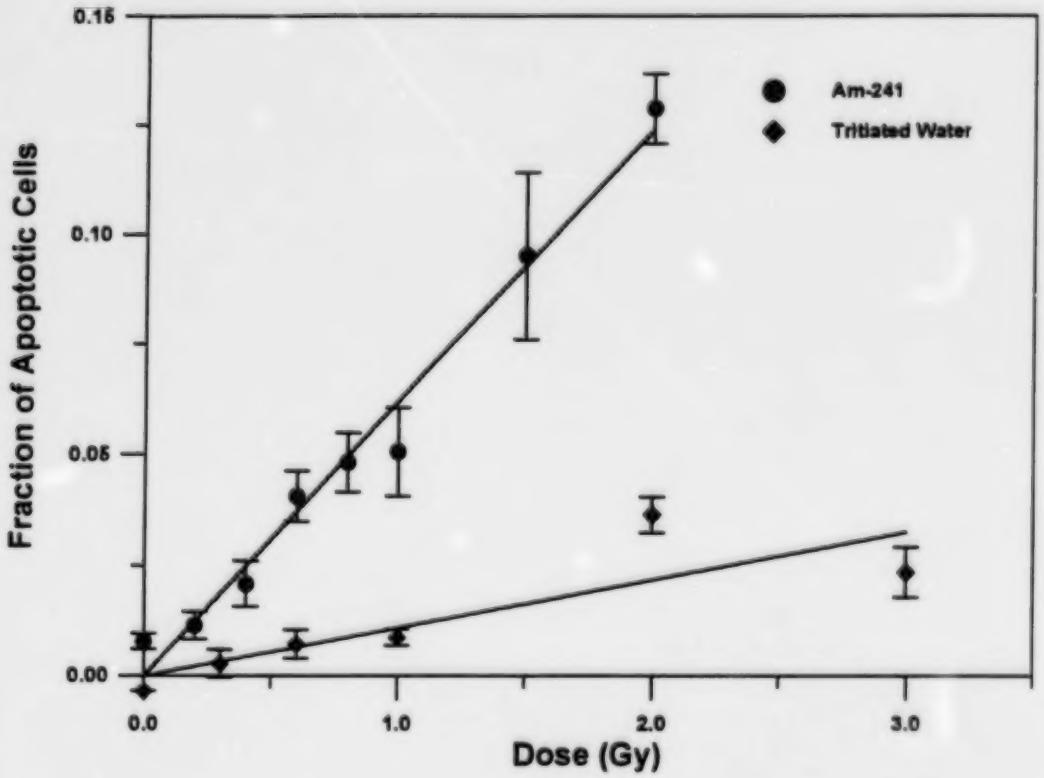

Figure 5: Fraction of apoptotic TK-6 cells 24 hours after ^{241}Am and $^{3}H_2O$. Error bars represent the standard error of the mean between experiments.

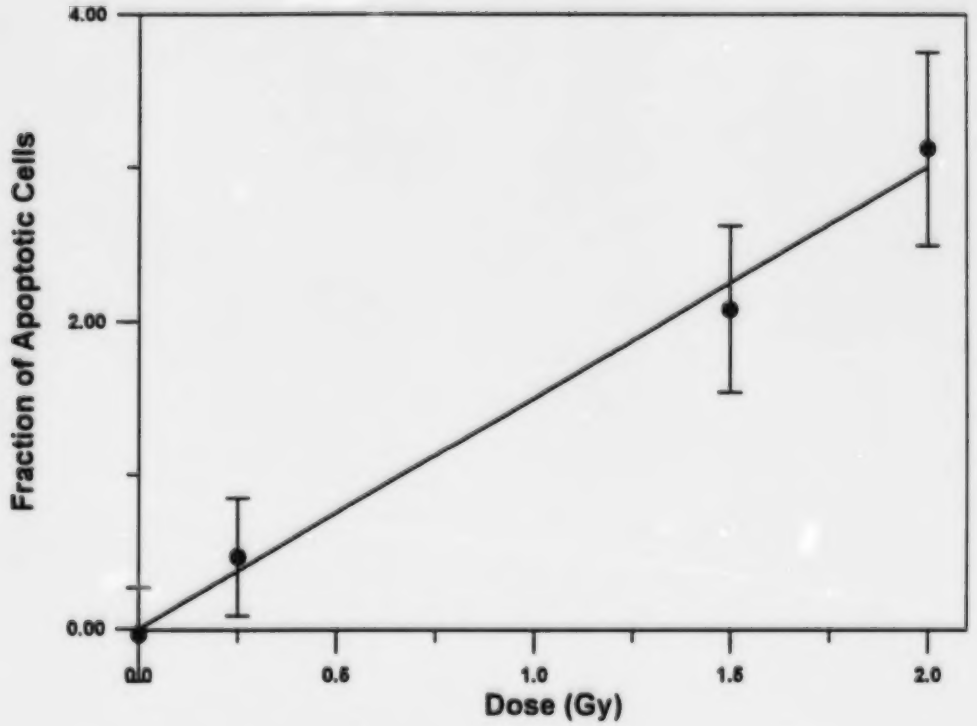

Figure 6: Fraction of apoptotic TK-6 cells 48 hours after irradiation with neutrons.

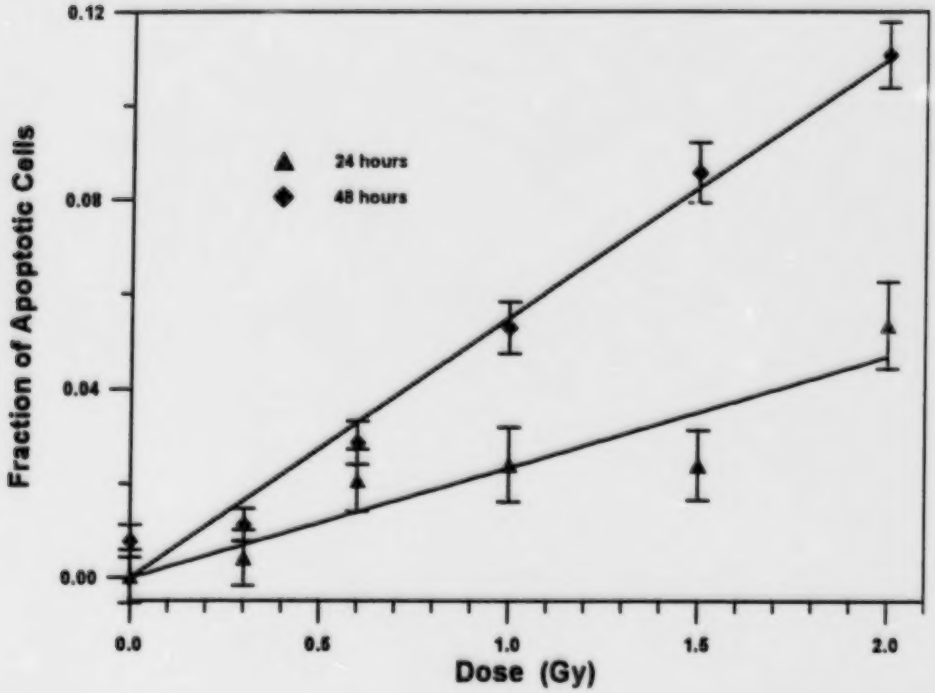

Figure 7: Fraction of apoptotic TK-6 cells 24 and 48 hours after irradiation with 250 kVp X-rays.

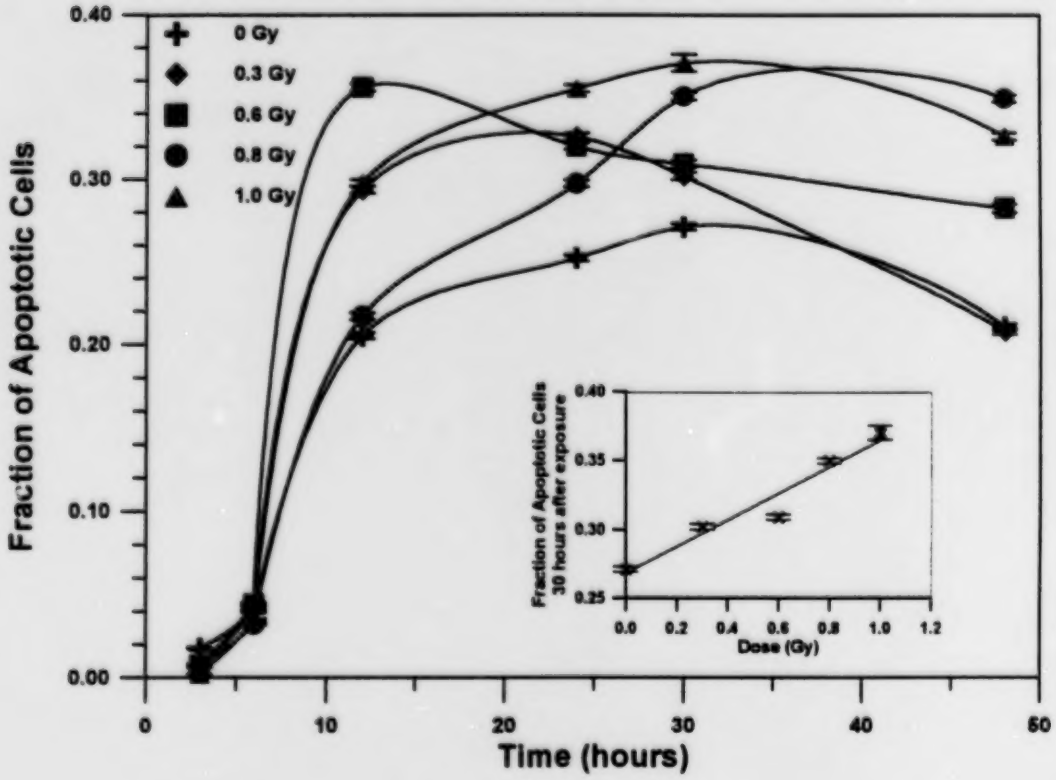

Figure 8: Fraction of apoptotic cells at various doses as a function of time on human whole blood irradiated with 190 kVp X-rays. Insert: Fraction of apoptotic cells 30 hours after exposure to 190 kVp X-rays as a function of dose.

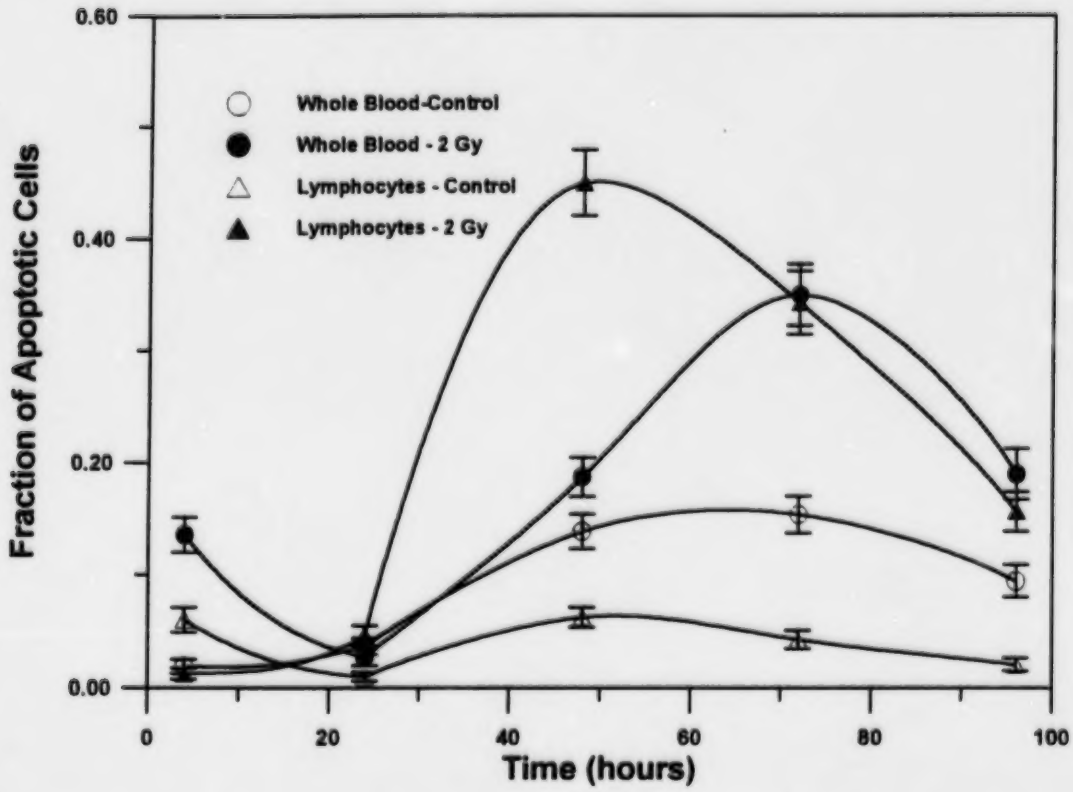

Figure 9: Fraction of apoptotic cells in whole blood and isolated white blood cells in a responding donor after 2 Gy of 250 kVp X-irradiation as a function of time after irradiation.

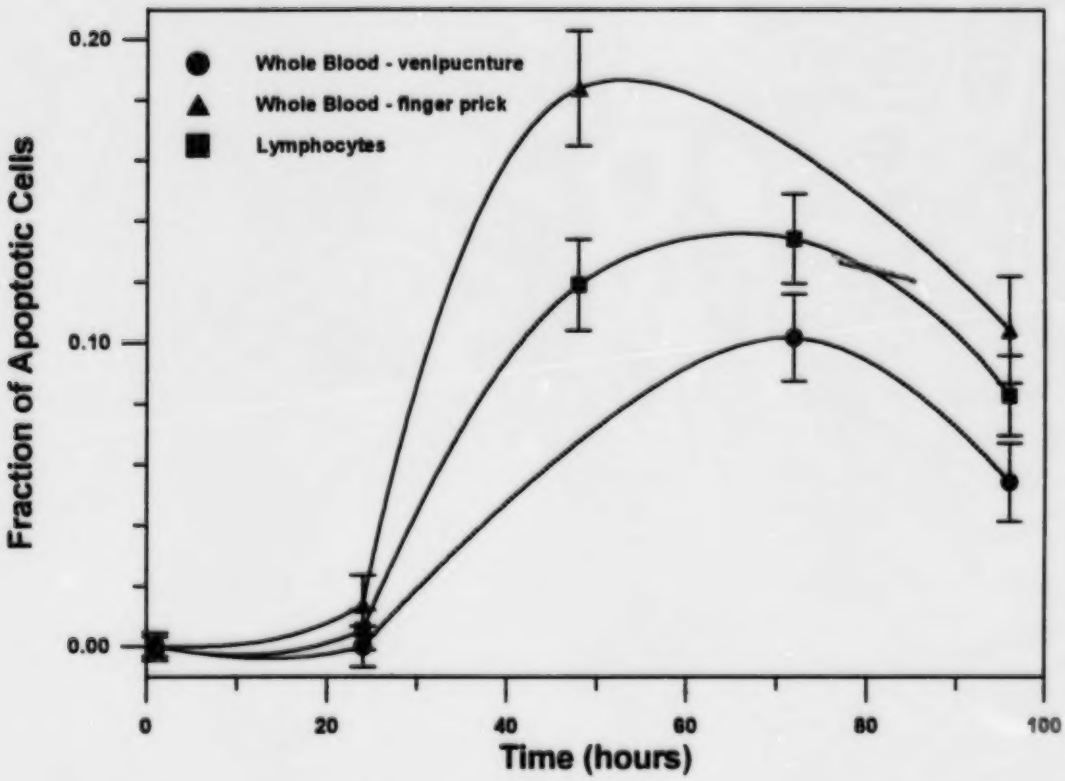

Figure 10: Fraction of apoptotic cells in isolated whole blood cells from the same donor obtained by either venipuncture or finger prick, after 2 Gy of 250 kVp X-irradiation as a function of time after irradiation.

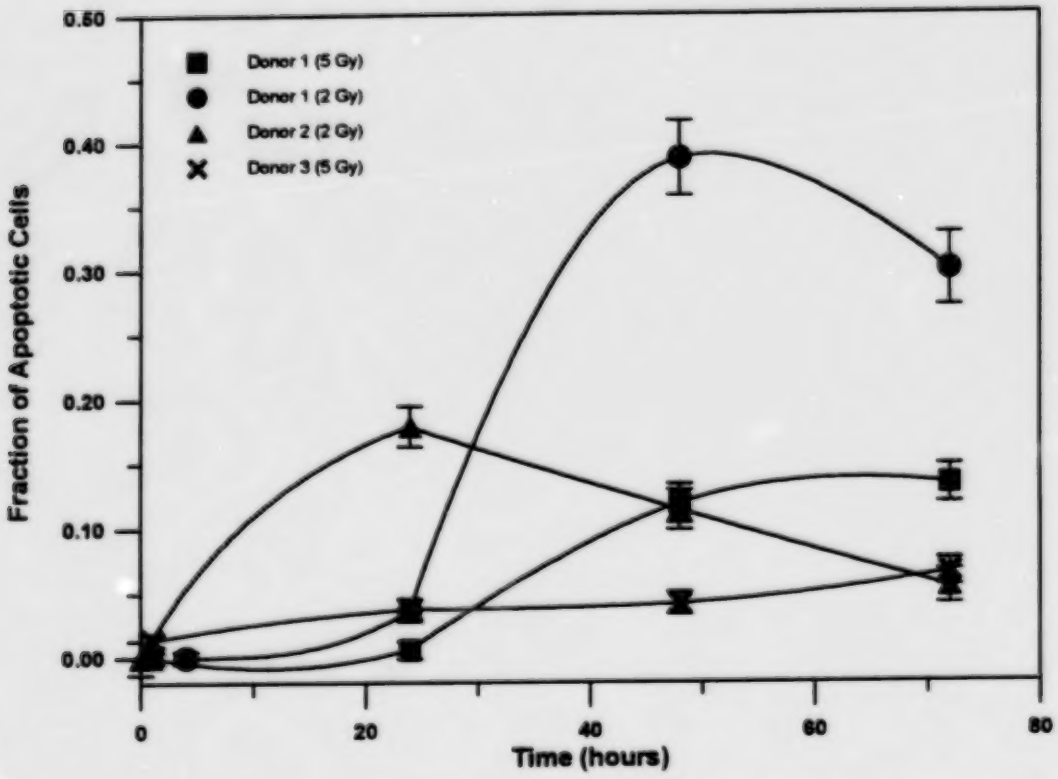

Figure 11: Fraction of apoptotic cells in white blood cells from three donors exposed to 2 or 5 Gy of 250 kVp X-rays.

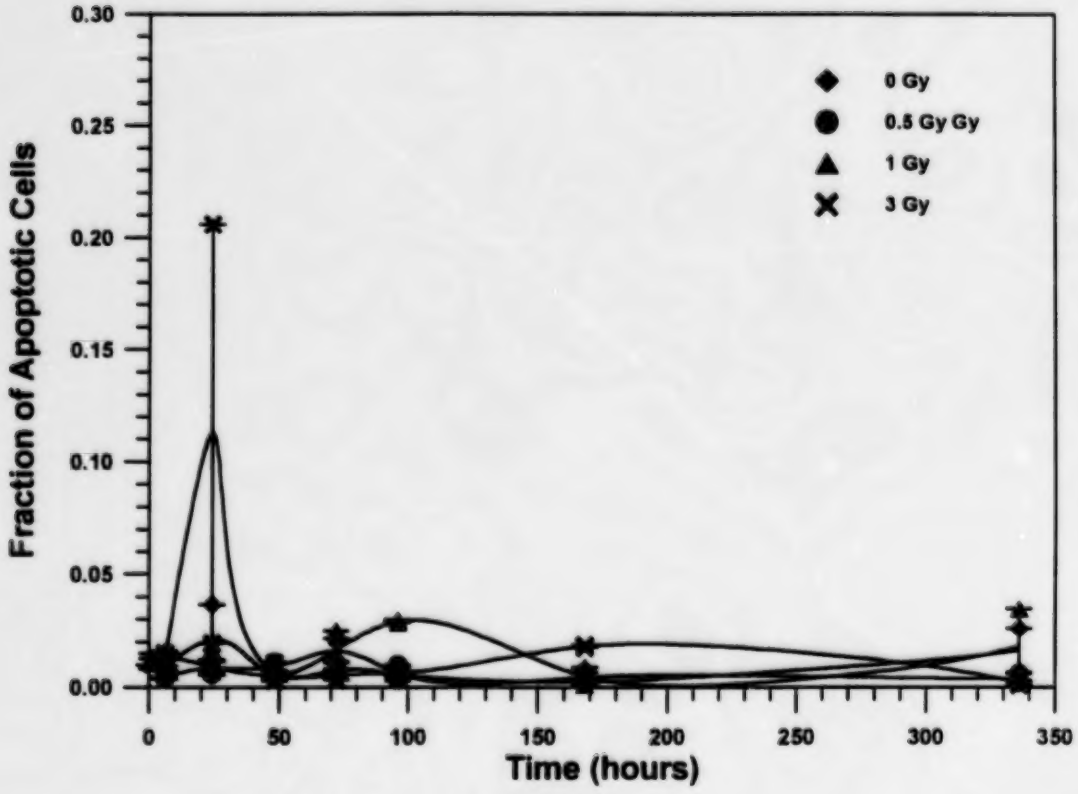

Figure 12. Fraction of apoptotic cells as a function of time in whole blood isolated from mice irradiated with 0 or 3Gy 120 kVp X-rays *in vivo*.

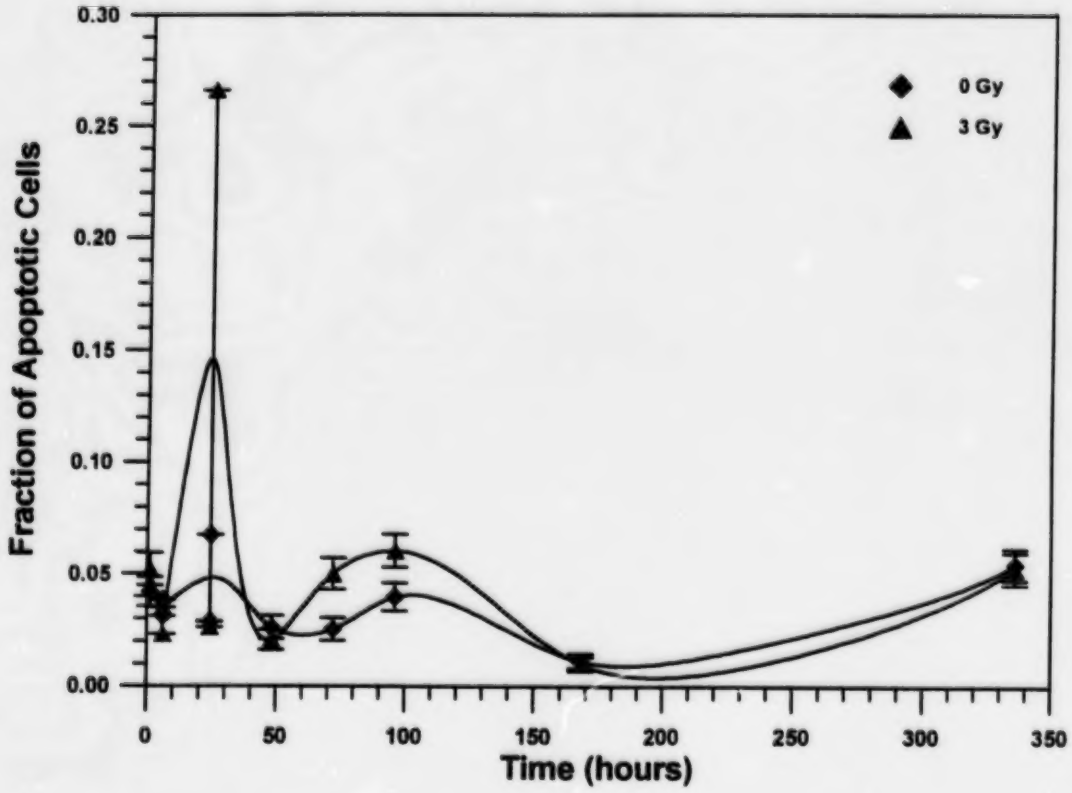

Figure 13. Fraction of apoptotic cells as a function of time in testes isolated from mice irradiated with 0 or 3Gy 120 kVp X-rays *in vivo*.

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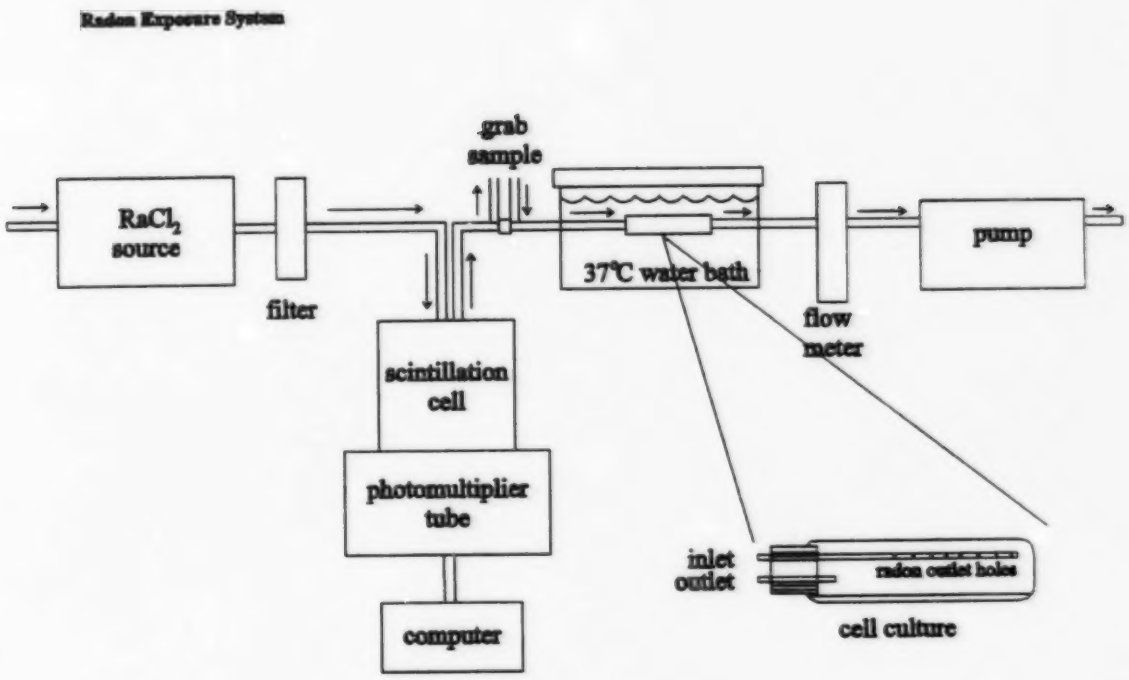
APPENDIX A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